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Spoilage of Blood Sausages *Morcilla de Burgos* Treated with High Hydrostatic Pressure

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Running title: Spoilage of *Morcilla de Burgos*

ABSTRACT

In this study, the microbial ecology of the blood sausages *morcilla de Burgos*, subjected to high hydrostatic pressure treatment (HPP), was studied by culture-dependent and -independent methods. *Morcilla de Burgos* is the most traditional and famous blood sausage in Spain. The producers are interested in extending its shelf-life in order to expand their market and to reduce losses attributed to spoilage. Sausage batter prior to stuffing and blood sausages HPP treated or not (control) were analyzed at 0, 9, 14, 21, 28 and 35 days of storage at 4°C. Lactic acid bacteria, *Pseudomonas* spp. and aerobic mesophilic bacteria were investigated by traditional plating. PCR-denaturing gradient gel electrophoresis (DGGE) was used to analyze the DNA and the RNA extracted directly from the blood sausages, as well as bulk cells of LAB and *Pseudomonas* spp. The results showed that HPP improved the shelf life of *morcilla de Burgos* to 28 days in comparison with control samples. The populations responsible for spoilage, namely LAB, remained lower in HPP treated samples when compared with the control samples. Only at 35 days of storage they reached values of 10^8 cfu/g, leading to the spoilage of the product. Although, HPP affected the LAB population, they were able to recover the injury provoked by the treatment. Lastly, HPP seemed to affect differently LAB species detected. While *Leuconostoc mesenteroides* was completely inactivated by HPP, *Weissella viridescens* was able to recover and carry out the typical spoilage of the product. *Pseudomonas* spp. remained under detection level ($<10^2$ CFU/g) after the HPP treatment.

Keywords: spoilage; *morcilla de Burgos*; culture-independent methods; PCR-DGGE; RT-PCR-DGGE

1. Introduction

Morcilla de Burgos is a popular cooked blood sausage produced in the region around Burgos, in the north of Spain. It is made by a mixture of onion, rice, animal fat (mainly lard), blood and different spices that included among others paprika, black pepper and oregano according to the local producer, stuffed in natural pork or beef casings and boiled for about 1 h at 90–95ºC, air cooled to 8–10ºC and finally chilled stored at 4ºC. The typical way to consume this product is by deep frying in vegetable oils, roasted or boiled as part of other traditional dishes. Physicochemical and sensory characteristics of this product have been described in a previous work (Santos et al., 2003). When *morcilla* is packaged under vacuum or modified atmosphere, the typical spoilage microflora of aerobic storage, predominantly *Pseudomonas* spp., is replaced by growth of lactic acid bacteria (LAB) (Santos et al., 2005a). Shelf-life of vacuum packaged *morcilla de Burgos* is from 14 up to 21 days depending on the initial contamination and the storage conditions (Santos et al., 2005a). After this time, presence of slime, vacuum loss, sour odour and taste are readily noticed in the product. Cooked meat products, as *morcilla de Burgos,* are liable to contamination during post-cooking handling, particularly during the chilling step, just before vacuum packaging. Producers are interested in extending the shelf-life of this product in order to increase the potential market and satisfy the consumer. Consumer demands are currently driven towards more natural food and as a consequence producers and scientists are searching for more natural antimicrobial preservatives and new mild technologies such as high pressure treatments (Hugas et al., 2002).

High hydrostatic pressure treatment (HPP) is a non-thermal mild technology of growing interest since pressures between 300 and 600 MPa inactivate yeasts, moulds and most

vegetative bacteria, including most spoilage and pathogenic bacteria, but vitamins, colour and flavour remain largely unaffected (Hugas et al., 2002). The inactivation of microorganisms by HPP is probably the result of a combination of factors, so cell death is due to multiple or accumulated damage inside the cell like destabilization of the structural and functional integrity of the cytoplasmic membrane, protein denaturation and inhibition of genetic mechanisms (Hoover et al., 1989; Simpson and Gilmor, 1997). The efficiency of the treatment is influenced by the pressure level achieved, temperature and exposure time as well as pH, type of microflora or food composition (Garriga et al., 2004). The ability of bacteria to survive HPP can be greatly increased when treated in nutritionally rich media like meat or meat products which contain carbohydrates, proteins and fat. For this reason, it is recommended to test microbial resistance within real food matrices (Garriga et al., 2004; Park et al., 2001). High pressure treatments have been successfully applied to meat and meat products contributing to the maintenance of sensorial properties and extending the shelf-life (Garriga et al., 2004; Yuste et al., 1998).

Due to the known limitations of conventional microbiological methods, characterization of microorganisms which require selective enrichment and subculturing is problematic or impossible. Moreover, in the last decade it was been shown that classical microbial techniques do not accurately detect microbial diversity (Ben Omar and Ampe, 2000; Hugenholtz et al., 1998). In the early 90s, a culture-independent method, termed denaturing gradient gel electrophoresis (DGGE), was developed, that has the potential to study the microbial population quickly and economically (Muyzer et al., 1993). The trend to move towards methods that avoid the use of selective cultivation and isolation of bacteria from natural samples is justified, considering the biases related to traditional culture-dependent methods. As a matter of fact, different authors described tremendous

differences between isolated and naturally occurring species present in various habitats (Head et al., 1998; Hugenholtz et al., 1998; Hugenholtz and Pace, 1996; Pace, 1997). DGGE has been extensively used to study the microbial ecology of fermented foods (Cocolin et al., 2001; Ercolini et al., 2003; Mills et al., 2002) and its use in the field of food microbiology has been recently reviewed by Ercolini (2004). Moreover, in the last years, studies focusing on the use of DGGE to follow the spoilage process in fresh meat have been published (Ercolini et al., 2006; Fontana et al., 2006). By using DNA as molecular marker it is possible to determine the presence or absence of a particular bacterial species in the sample, but it is not possible to assure that these bacteria are alive or dead. In that sense, the use of RNA can help in the understanding of the alive and active population within a microbial ecosystem.

The aim of the present study was to follow the spoilage bacterial population in *morcilla de Burgos*, and evaluate their behavior when a HPP treatment was applied to extend their shelf-life.

2. Materials and Methods

2.1 Samples and processing of the blood sausages

Morcillas stuffed in natural beef casings prepared by one producer were selected for the different preservation experiments. The ingredients in the factory included raw onion (54%), rice (17%), lard (17%), blood (8%), salt (1.8%) and a mixture of different spices that included paprika, black pepper and oregano (2.2%). The chopped onion and fat were mixed with the rice, salt, spices and blood and the sausage emulsion was stuffed into 35-45 mm natural beef casings that were preserved with salt and rinsed in clean water before use. No nitrite was included in the formulation. The blood sausages were then transferred to a cooking container and boiled in water at 95-96ºC for around one hour, air cooled to 8–10ºC, and vacuum packaged. Blood sausages were subjected to high pressure at 600 MPa for 10 min, as previously suggested by Borek et al. (2002). A discontinuous hydrostatic pressurization unit Wave 6000/135 (NC Hyperbaric, Burgos, Spain), capable of operating up to 600 MPa, with a cylinder measuring 0.30 m in diameter and 2.20 m in length, and with a working volume of 0.135 m^3, was used. The pressure transmission fluid was water. Initial water temperature was 15ºC, increasing around 3ºC per 100 MPa during high pressure processing due to adiabatic heating. Water temperature after HPP was 17ºC. The blood sausages were stored in a dark place at 4ºC.

Samples of sausage batter prior to stuffing, HPP treated and not treated (control) blood sausages vacuum-packaged, were analyzed in triplicate at 0, 9, 14, 21, 28 and 35 days of storage at 4°C.

2.2 pH measurements

Potentiometric measurements of pH were made with a pin electrode of a pH meter (pH M82; Radiometer Copenhagen, Cecchinato, Italy) inserted directly into the sample. Three independent measurements were obtained on each sample. Means and standard deviations were calculated.

2.3 Microbiological analysis

The samples were subjected to microbiological analysis to monitor the dynamic changes of the spoilage populations during storage of the blood sausages treated or not by high pressure. In particular, A slice of 20 g of *morcilla,* (casing included), was sterile weighted, diluted in 80 ml of saline-peptone water (8 g of NaCl/liter, 1 g of bacteriological peptone/liter [Oxoid, Milan, Italy]), and homogenized for 120 s in a stomacher machine (PBI, Milan, Italy), prior to the preparation of 1/10 serial dilutions for microbiological analysis. The following microbial parameters were determined on duplicate agar plates: aerobic mesophilic bacteria (AMB) plated in Gelisate agar (Oxoid) and incubated at 30ºC for 48 hours; Lactic Acid Bacteria (LAB), grown in MRS agar (Oxoid) and incubated anaerobically in 6% CO_2 at 30ºC for 48 hours; *Pseudomonas* spp., plated on Pseudomonads agar (Oxoid) supplemented with CFC (Cetrimide, Fucidine, Cefaloridine, Oxoid) and incubated at 25ºC for 48 hours. After counting, means and standard deviations were calculated.

2.4 DNA extraction from bulk cells

Bulk cells (Ercolini et al., 2001) of LAB and *Pseudomonas* spp. were prepared by scraping cultures from nutrient agar plates with counts between 30 – 300 colonies and resuspended in 1.5 ml of sterile saline peptone water (8 g of NaCl/liter, 1 g of

bacteriological peptone/liter; [Oxoid]). Colonies were homogenized and 150 µl of this microbial cell suspension, were centrifuged at 4 ºC for 10 min at 14,000 x *g* to pellet the cells, which were resuspended in 150 µl of proteinase K buffer (50 mM Tris-HCl, 10 mM EDTA [pH 7.5], 0.5% [wt/vol] sodium dodecyl sulfate). Twenty-five microliters of proteinase K (25 mg/ml; [Sigma, Milan, Italy]) were added, and a 50°C treatment was performed for 1.5 h. After this step, 150 µl of 2x breaking buffer (4% [vol/vol] Triton X-100, 2% [wt/vol] sodium dodecyl sulfate, 200 mM NaCl, 20 mM Tris [pH 8], 2 mM EDTA [pH 8]) were added and the solution was moved into a screw cap tube containing 0.3 g of glass beads. Three hundred microliters of phenol-chloroform-isoamyl alcohol (25:24:1, pH 6.7; [Sigma]) were added and the tubes were subjected to three 30-s treatments at the maximum speed, with an interval of 10 s each, in a bead beater (Mini Bead Beader 8; Biospec Products, Inc., Bartlesville, Okla.). Then, 300 µl of TE were added (10 mM Tris [pH 8], 2 mM EDTA [pH 8]) and the DNA, in the aqueous phase was recovered and precipitated with ice-cold ethanol after centrifugation at 12,000 x *g* at 4°C for 10 min. The DNA was collected at 14,000 x *g* at 4°C for 10 min. Fifty microliters of sterile water was added, and a 30-min period at 45°C was used to facilitate the nucleic acid solubilization. One microliter of DNase-free RNase (Roche Diagnostics, Milan, Italy), was added to digest RNA by incubation at 37°C for 1 h.

2.5 Direct extraction of nucleic acids from blood sausages

From each sampling point, 20-g samples, were homogenized in 80 ml of saline peptone water (8 g of NaCl/liter, 1 g of bacteriological peptone/liter; [Oxoid]) and 40 ml of solution were transferred into a 50 ml sterile tube. Big debris was allowed to deposit for 5 min, and 4 ml of supernatant was split into two 2-ml aliquots in tubes, one for DNA and one for RNA extraction. They were subjected to centrifugation at 4°C for 10 min at

14,000 x *g* to pellet the cells, which were subjected to DNA and RNA extraction. The DNA was extracted as described for the bulk cells, while for the RNA 300 µl of phenol-chloroform (5:1, pH 4.7; [Sigma]) were added before the bead beater treatment. The DNA and RNA were collected at 14,000 x *g* at 4°C for 10 min, after addition of 1 ml ice-cold ethanol. Fifty microliters of sterile water were added, and a 30-min period at 45°C was used to facilitate the nucleic acid solubilization. One microliter of DNase-free RNase (Roche Diagnostics) and 1 µl of RNase-free DNase (Roche Diagnostics) were added to digest, respectively, RNA and DNA by incubation at 37°C for 1 h.

2.6 Reverse transcription step

Reverse transcription (RT)-PCR was performed with the RevertAid^{TM} M-MuLV reverse transcriptase (Promega, Milan, Italy). One µl of total RNA (approximately 0.1 µg) was mixed in 10 µl of DNase, RNase-free sterile water containing 10 µmol of the P2 primer (5'-TTC CCC ACG CGT TAC TCA CC-3') (Klijn et al., 1991), and incubated at 70°C for 5 min. Immediately after chilling in ice, a mixture of 25 mM Tris HCl pH 8.3, 25 mM KCl, 2 mM $MgCl_2$, 5 mM DTT, 1 mM each dNTPs and 20 UI ribonuclease inhibitor (Promega) were transferred in the reaction tube. After 5 min at 37°C, 1 µl reverse transcriptase was added followed by an incubation at 42°C for 60 min and at 70°C for 10 min treatment to stop the reaction. Two µl of the synthesized cDNA were used for the PCR amplification.

2.7 PCR protocol

Amplifications of DNA and cDNA were carried out with primers P1, 5'-GCG GCG TGC CTA ATA CAT GC-3', and P2, 5'-TTC CCC ACG CGT TAC TCA CC-3' (Klijn et al., 1991), in a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl,

1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (dNTPs), 1.25 U of *Taq* polymerase (Applied Biosystems, Milan, Italy) and 0.2 µM concentrations of each primer. Two microliters of template DNA (50 ng total) and cDNA were added to the mixture. Amplifications were carried out in a PTC200 thermal cycler (Biorad, Hercules, Calif.) in a final volume of 50 µl by using an amplification characterized by one cycle of 95ºC for 5 min, then 30 cycles of denaturation of 95°C for 1 min, annealing at 50 ºC for 45 sec and extension at 72°C for 1.5 min. A final extension of 72°C for 5 min ended the amplification cycle. Agarose gel electrophoresis (2% w/v) in TBE buffer containing 0.5 µg/ml ethidium bromide (Sigma) was used to assess the presence of the PCR product. A GC clamp (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3') was added to primer P1 when used for DGGE analysis (Sheffield et al., 1989).

2.8 DGGE analysis

The Dcode universal mutation detection system (Bio-Rad, Hercules, Calif.) was used for DGGE analysis. For PCR products obtained with the primers P1-P2, electrophoresis was performed in a 0.8-mm-thick polyacrylamide gel (8% [wt/vol] acrylamide-bisacrylamide [37.5:1]) with a denaturant gradient from 40 to 60% (100% corresponded to 7 M urea and 40% [wt/vol] formamide) increasing in the direction of the electrophoretic run. Gels were subjected to a constant voltage of 120 V for 4 h at 60°C, and after the electrophoresis, they were stained for 20 min in 1.25X Tris-acetate-EDTA containing 1X SYBR Green (final concentration; Molecular Probes, Eugene, Oreg.). They were visualized under UV, digitally captured, and analyzed by using the GeneGenius BioImaging System (SynGene, Cambridge, United Kingdom) for the recognition of the bands present.

2.9 Sequencing of DGGE bands and sequence analysis

Blocks of polyacrylamide gels containing selected DGGE bands were punched by sterile pipette tips. The blocks were then transferred in 50 µl of sterile water, and the DNA of the bands was left to diffuse overnight at 4°C. Two microliters of the eluted DNA were used for the reamplification and DGGE was run to confirm the presence of a single band with identical migration profile with respect to the one excised from the samples. After DGGE analysis, the PCR product was cloned into pGEM vector (Promega) and at least five clones were tested again by PCR-DGGE to check the mobility of the insert. When the migration was again identical to the original band cut, the clone was sent for sequencing to a commercial facility (MWG Biotech, Germany). The Blast program (Altschul et al., 1997) was used for the analysis of the sequences obtained.

3. Results

3.1 pH measurements

The results of the pH measurements are shown in Figure 1. Initial pH values were above 6.35 for all different samples. Control samples were characterized by a drop in pH values from pH above 6.20 (day 9) to pH just above 5.00 (day 21). No differences were detected from day 21 till the end. In the case of HPP samples, no decrease was observed during the first 28 days showing a pH over 6.0. Only at the end of the study (35 days), pH decreased dramatically reaching values around 5.40.

3.2 Conventional microbiological analysis

Results of plate counts obtained from sausage batter prior to stuffing and cooking showed a high number of bacterial populations: 10^7 colony forming units (cfu)/g of AMB on gelisate agar; 10^9 cfu/g of LAB and 10^6 cfu/g of *Pseudomonas* spp. (data not shown). Figure 2 shows the trends of the different microbial populations, monitored during storage, in control and HPP treated samples. Regarding the control, after the cooking step, the bacterial populations dramatically decreased and at day zero counts of about 10^3 cfu/g were only detected for AMB. The rest of the microbial populations examined were below the detection limit ($<10^2$ cfu/g). A considerable increase for all kinds of bacteria tested was observed after 9 days of storage. AMB reached a population of 10^6 cfu/g from day 9 till day 21 and only at the end of the study they decreased reaching values around 10^3 cfu/g. A progressive increase in LAB counts was observed from day 9, reaching values of 10^8 cfu/g, at the end of the study. *Pseudomonas* spp. counts did not show differences during the period monitored, being around 10^4 cfu/g from day 9 to day 28, although they slightly decreased at day 35.

In HPP treatments samples, AMB were of about 10^3 cfu/g at day zero, while the rest of microbial parameters studied were kept below the detection limit (<10^2 cfu/g). Although the counts for AMB increased to 10^4 cfu/g during the first week of storage, no differences were detected until day 28. Only at the end of the storage period a decrease in the counts was observed. LAB count showed progressive increase during cold storage reaching values over 10^8 cfu/g only at the end of storage. *Pseudomonas* spp. were always below the detection limit (<10^2 cfu/g), during the chilled storage.

3.3 DGGE profiles in morcilla samples

Morcilla samples were analyzed by PCR and RT-PCR-DGGE and the profiles obtained are shown in Figures 3 and 4, respectively. In Figures 5 and 6, the DGGE profiles of the LAB and *Pseudomonas* spp. bulks are presented, respectively. The identification of the bands, cut from the DGGE gels, by 16S rRNA gene sequencing is also reported in Table 1.

Concerning the bacterial ecology as determined by DNA-DGGE profiles (Fig. 3), species of *Weissella confusa*, *Weissella viridescens*, *Gamma proteobacterium* and *Leuconostoc lactis* were identified in sausage batter, HPP treated and control samples. While *W. confusa, W. viridescens* and *G. proteobacterium* were detected in all the samples, treated or not, *L. lactis* was detected, although as faint bands, at 0 and 9 days of storage in control samples and from 0 to 28 days in HPP treated samples. *Brochotrix thermosphacta* and *Leuconostoc mesenteroides* were only identified in control samples, indicating that these species could be more sensitive to high pressure treatments. *B. thermosphacta* and *L. mesenteroides* were detected from 14 to 28 days and from 14 to 35 days of storage, respectively. Also a band identified as *Oryza sativa* was detected only at day zero in the control sample.

The same species of bacteria were detected in the RNA-DGGE gels (Fig. 4), however their presence and appearance were rather different from those obtained by DNA-DGGE. In control samples, *L. lactis* and *W. confusa* were detected in the sausage batter and at day zero and at day 9 a third band appeared, identified as *B. thermosphacta*. *L. mesenteroides* showed an intense band from day 14 till day 35. It is remarkable that this species expressed higher band intensity when pH dropped from 6.20 on day 9 to 5.59 on day 14. *W. viridescens* band appeared from day 21 onwards. In HPP treated samples, *L. mesenteroides* band did not appear and pH dropped from 6.30 on day 28 to 5.39 on day 35 coinciding with the appearance of the band of *W. viridescens*. *L. lactis* showed an opposite behavior to *L. mesenteroides* and it seemed more resistant to HPP treatment according to the band pattern obtained: it was present from day 0 to day 28 and only at day 35 did it disappear. In the DNA and RNA-DGGE, different bands were visible in the upper part of the gels. They were all determined to be heteroduplexed by sequencing (data not shown).

3.4 DGGE profiles from bulk cells

The results obtained from the DGGE of the bulk cells from MRS and Psudomonas agar are shown in Figures 5 and 6 respectively. Concerning MRS agar apart from LAB, also others species, such as *Bacillus* sp., *Gamma proteobacterium*, and *Staphylococcus* sp., appeared at different sampling times. The species of LAB found in the DGGE gels were, however, the same as the ones detected directly in *morcilla* samples. *W. viridescens, W. confusa* and *L. mesenteroides* were identified in control and HPP samples. Similarly, in the bulks from the Pseudomonas agar with CFC supplement, non-*Pseudomonas* species, such as *Gamma proteobacterium, Enterobacteriaceae, W.*

confusa, W. viridescens and *Hafnia alvei* were detected. Also in this case the upper bands visible in the gels were defined by sequencing as heteroduplex (data not shown).

4. Discussion

Blood sausages are products difficult to preserve due to their rich nutrient composition, relatively high pH (up to 6.10), and high water activity with values over 0.984 (Santos et al., 2003). Although, high temperature during cooking process eliminates the vegetative forms of microorganisms and only bacterial spores survive the treatment, handling of the product during cooling, leads to post-cooking contamination by other bacteria on the surface of the product (Korkeala et al., 1987; Mäkelä and Korkeala, 1987; Santos et al., 2005a).

In order to follow precisely the microbial dynamics with traditional methods, the gelisate agar was used to monitor the viable aerobic bacteria. As described by other authors (Cocolin et al., 2004), this medium does not support the growth of LAB, thereby the counts obtained should refer only to other microbial groups found in the product during storage, other than LAB. Comparing the high microbial counts obtained for the batter, prior to stuffing and cooking, with the results obtained for the stuffed product, a reduction for all microbiological parameters studied were obtained (data not shown). LAB and pseudomonas counts were below the detection limit ($<10^2$ cfu/g) showing a reduction of about 5 and 4 orders of magnitude, respectively. Despite the low initial contamination, LAB became the predominant natural flora and they were responsible for the decrease in the pH of the product packaged under vacuum. They reached values above 10^7 cfu/g after 14-21 days of storage, while other microbial groups were restricted probably because of the competition with LAB (Korkeala and Björkroth, 1997; Samelis et al., 2000; Santos et al., 2005a).

The results presented here show that the high pressure treatment is effective in extending the shelf life of *morcilla de Burgos*, as already suggested by other authors (Borek et al., 2002; Diaz et al., 2006a). HPP treatment determined the reduction of the *Pseudomonas* spp. populations under the detection level ($<10^2$ cfu/g) after pressure treatment. HPP has been reported as being very effective in diminishing or eliminating gram negative bacteria (Linton et al., 2004; Tuboly et al., 2003), while gram positive bacteria are more resistant (Hugas et al., 2002; Smelt, 1998). Here, the LAB populations were not completely inactivated by the HPP treatment and at the end of the period monitored an increase in LAB counts, determining an intense drop of pH, was observed. The protective effects of the rough surface of the product, together with the fat content that sticks to the casing surface during the cooling step, probably are the causes of the resistance of LAB in *morcilla.* Other authors have reported even the complete inactivation of this microbial group in non-inoculated minced meat packaged in sterile polyvinylidene chloride tubing (casing) after high pressure treatment at 450 MPa for 20 min at 20ºC (Carlez et al., 1994), and in inoculated cooked ham with added nisin after high pressure treatment at 400 MPa for 10 min at 17ºC (Garriga et al., 2002).

Santos et al. (2005a, b) showed that the main spoilage bacteria in vacuum-packaged *morcilla de Burgos* are some species of heterofermentative LAB belonging to the genera *Weissella* and *Leuconostoc,* and this evidence was confirmed by the results obtained in this study by DNA and RNA-DGGE. After cloning and sequencing, some bands showed to belong to the same species, underlining the presence of multicopies of the 16S rRNA gene for these microorganisms, as already described by other authors (Cocolin et al., 2004; Rantsiou et al., 2005). HPP treatment produced relevant modifications in the growth dynamics of the principal spoilage bacterial populations. In

both, HPP treated and control product, the main spoilage bacteria were *W. viridescens, W. confusa, L. lactis* and *L. mesenteroides*, although they differed in prevalence. According to the results obtained, it seems that *L. mesenteroides* is more sensitive to high pressures, as already reported in other products (Basak et al., 2002; Tahiri et al., 2006), than *W. viridescens* that shows more resistance to high pressure (Diez et al., 2006a; Park et al., 2001). Santos et al. (2005b) obtained high survival of *W. viridescens* when *morcilla de Burgos* was pasteurized, after cooking and packaging. Smelt (1998) suggested that heat-resistant microorganisms are also more resistant to pressure and this evidence can explain the behavior of *W. viridescens* in HPP treated samples. A second species belonging to the genus *Weissella*, responsible for spoilage in *morcilla de Burgos*, was *W. confusa.* Other studies (Santos et al., 2005b) have described the detection and identification of this species in blood sausages. Björkroth et al. (2002) isolated it in Malaysian foods and in clinical samples from humans and animals, and Morishita and Shiromizu (1986) reported the presence of *W. confusa* in meat and meat products. Like *W. viridescens*, also *L. lactis* has been isolated from *morcilla de Burgos* (Santos et al., 2005b), from fermented sausages (Rodriguez et al., 1995), pork meat (Garver et al., 1993) and poultry meat (Barakat et al., 2000). *Pseudomonas spp.* disappear after HPP treatment. The significance of *Gamma proteobacterium* in this product is unknown.

Concerning the RNA-DGGE profiles, it is interesting to notice that bands related to *L. mesenteroides* in control samples, and to *W. viridescens* in HPP treated samples, appeared more intense at the time-point when pH dropped in *morcilla*. In that sense, it is possible that HPP treatment affected some LAB, namely *W. viridescens*, that showed an increase in the activity only at day 35, after recovering from the damage caused by the

HPP treatment. *L. lactis* showed a very interesting behavior in control and HPP samples. It seemed that it was present in the product until the pH started to drop (Fig. 4) and this evidence may reflect the high sensitivity of this species to pH changes. The same picture was observed in the HPP treated samples between day 28 and 35, as well.

With respect to the bacterial ecology determined by bulk DGGE profiles from MRS agar and Pseudomonas agar, the main result highlighted was the non-selectivity of the media used. Moreover, the analysis of the bulk cells prepared from the MRS plates emphasized once more the different picture in the ecology that can be obtained by using culture-dependent and -independent methods. By RNA-DGGE analysis it was determined that in HPP treated samples *W. viridescens* was active only at the end of the storage period (Fig. 4), while on the plates it could be detected already from day 14 (Fig. 5).

In summary, high pressure treatment is effective in extending the shelf life of *morcilla de Burgos* to 28 days in comparison with control samples. As determined by traditional plating, the populations responsible for spoilage, namely LAB, remain significantly lower in HPP treated samples when compared with the control samples. Only at 35 days of storage they reached values of 10^8 cfu/g, leading to the spoilage of the product. Moreover, the HPP treatment was responsible for the change in the ecology of the product during storage. High pressure seemed to affect differently LAB species detected in the blood sausages. While *L. mesenteroides* was completely inactivated by HPP, *W. viridescens* was able to recover and carry out the typical spoilage of the product.

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Table 1. Identification of the bands excised from DGGE gels achieved by sequencing and alignment in Gene Bank by using the BLAST program (Altschul et al., 1997).

Bands[a]	Size (bp)	Closest relative	%Identity	Source[b]
From DNA:				
1	121	*Weissella confusa*	100	M23036
2	121	*Weissella viridescens*	100	AB023236
3	89	*Gamma proteobacterium*	80.1	AF505726
4	78	*Oryza sativa*	100	AC078977
5	94	*Leuconostoc lactis*	100	DQ682974
6	93	*Brochotrix thermosphacta*	100	AY543029
7	94	*Leuconostoc mesenteroides*	100	DQ523483
From RNA:				
8	94	*Leuconostoc lactis*	100	DQ682974
9	93	*Brochotrix thermosphacta*	100	AY543029
10	94	*Leuconostoc mesenteroides*	100	DQ523483
11	89	*Gamma proteobacterium*	80.1	AF505726
12	121	*Weissella confusa*	100	M23036
13	121	*Weissella viridescens*	100	AB023236
14	89	*Gamma proteobacterium*	80.1	AF505726
Bulk cells MRS:				
15	121	*Weissella confusa*	100	M23036
16	89	*Gamma proteobacterium*	80.1	AF505726
17	94	*Leuconostoc mesenteroides*	100	DQ523483
18	89	*Gamma proteobacterium*	80.1	AF505726
19	121	*Weissella viridescens*	100	AB023236
20	93	*Staphylococcus* sp.	100	DQ628968
21	91	*Bacillus* sp.	98.8	AY462215
22	121	*Weissella viridescens*	100	AB023236
23	94	*Leuconostoc mesenteroides*	100	DQ523483
Bulk cells CFC:				
24	89	*Gamma proteobacterium*	80.1	AF505726
25	89	*Pseudomonas* sp.	100	DQ530082
26	121	*Weissella confusa*	100	M23036
27	121	*Weissella viridescens*	100	AB023236
28	95	*Enterobacteriaceae* bacterium	98.9	DQ490333
29	95	*Hafnia alvei*	100	AY572428

[a]Bands numbered as indicated on DGGE gels shown in Figures 3, 4, 5 and 6.

[b]Accession number of sequence of closest relative found with Blast search.

Figure legends.

Figure 1. pH trends of the samples monitored in this study.

Figure 2. Microbial population dynamics monitored in this study, as determined by classical methods. Abbreviations: AMB, aerobic mesophilic bacteria; LAB, lactic acid bacteria.

Figure 3. DNA DGGE profiles. The numbers represent the bands that were cut and subjected to sequencing. Lane designations indicate the treatment (M: sausage batter; C: control; T: HPP treatment) and the time of sampling. H, heteroduplex bands. Numbers from 1 to 7 correspond to species listed in Table 1.

Figure 4. RNA DGGE profiles. The numbers represent the bands that were cut and subjected to sequencing. Lane designations indicate the treatment (M: sausage batter; C: control; T: HPP treatment) and the time of sampling. H, heteroduplex bands. Numbers from 8 to 14 correspond to species listed in Table 1.

Figure 5. LAB bulk DGGE profiles. The numbers represent the bands that were cut and subjected to sequencing. Lane designations indicate the treatment (M: sausage batter; C: control; T: HPP treatment) and the time of sampling. H, heteroduplex bands. Numbers from 15 to 23 correspond to species listed in Table 1.

Figure 6. *Pseudomonas spp.* bulk DGGE profiles. The numbers represent the bands that were cut and subjected to sequencing. Lane designations indicate the treatment (M: sausage batter; C: control) and the time of sampling. H, heteroduplex bands. Numbers from 24 to 29 correspond to species listed in Table 1.

Figure 1.

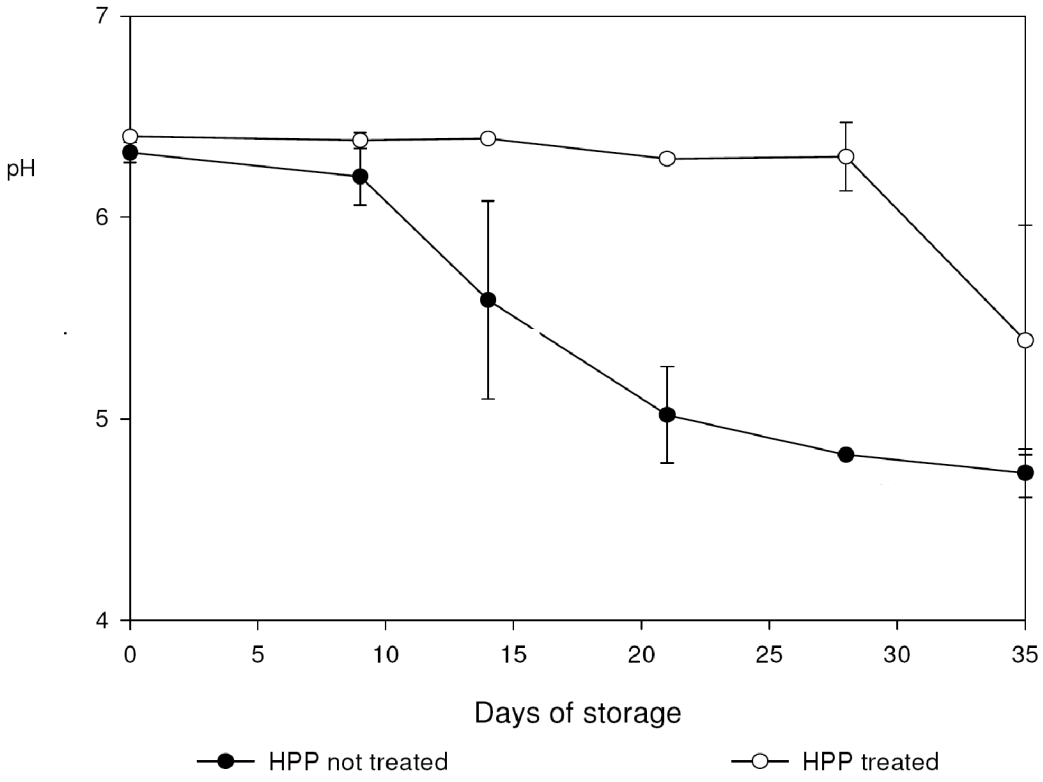
pH
7
6
5
4
0
5
10
15
20
25
30
35
Days of storage
HPP not treated
HPP treated

Figure 2.

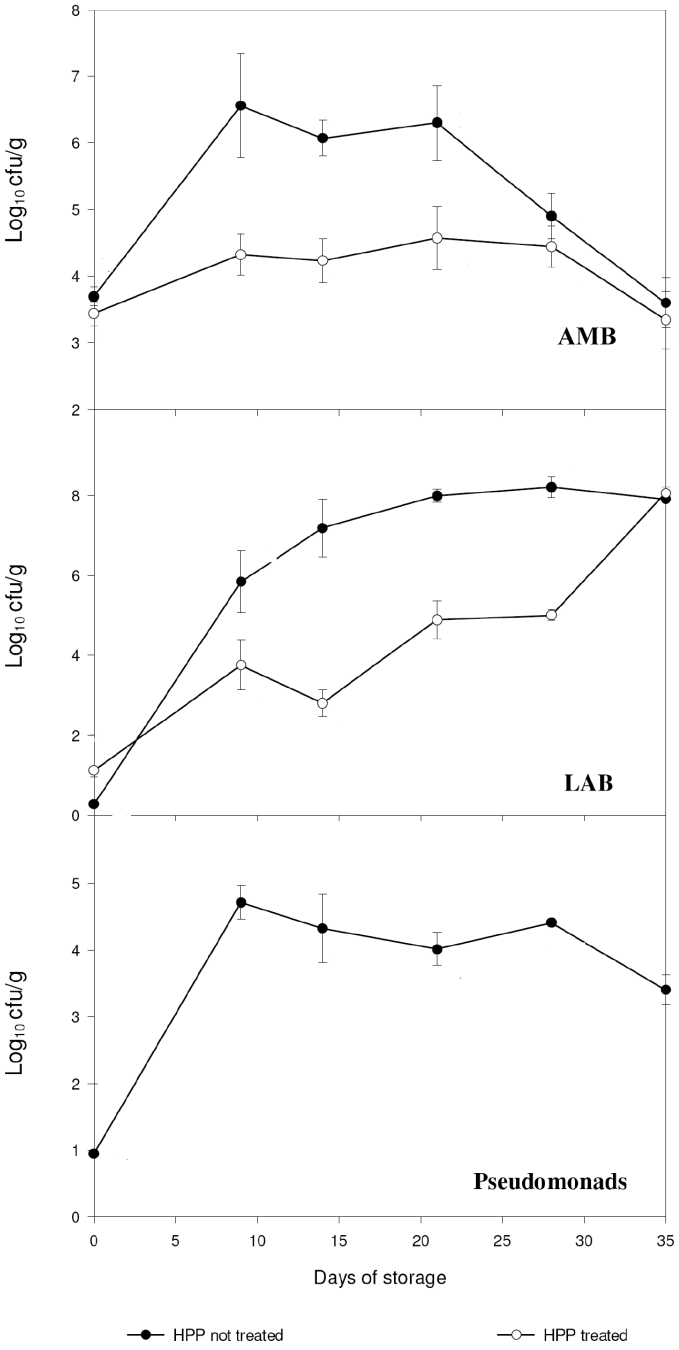
Log10 cfu/g
AMB
LAB
Pseudomonads
Days of storage
HPP not treated
HPP treated

Figure 3.

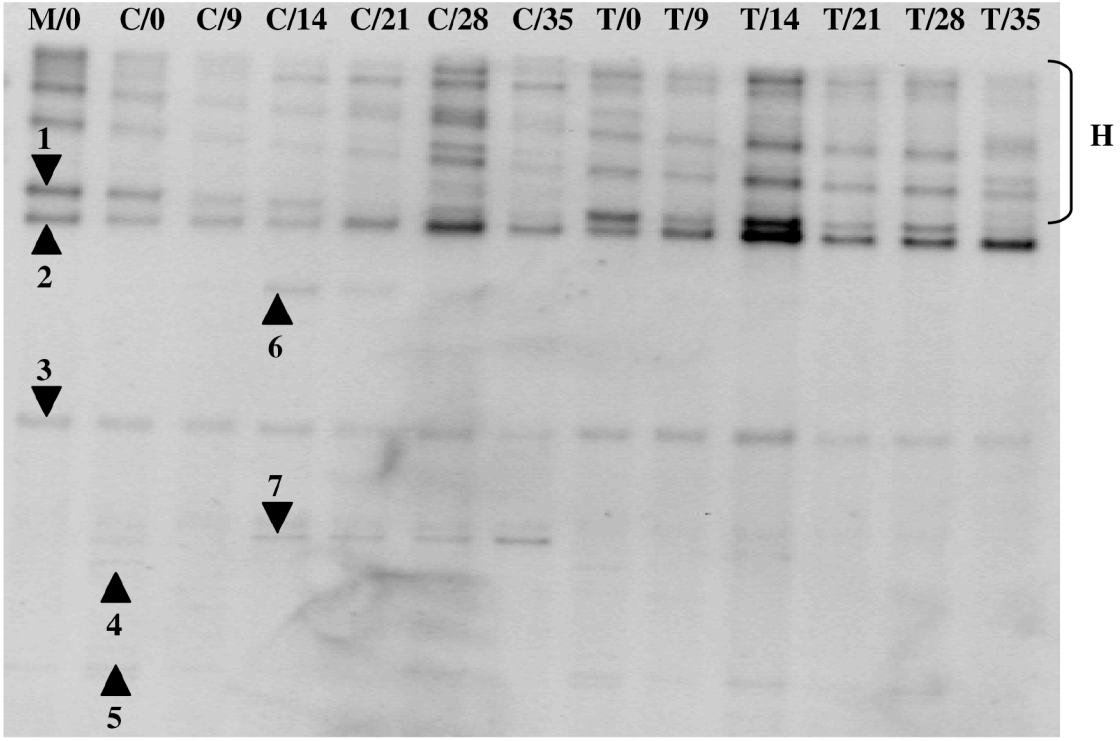

Figure 4.

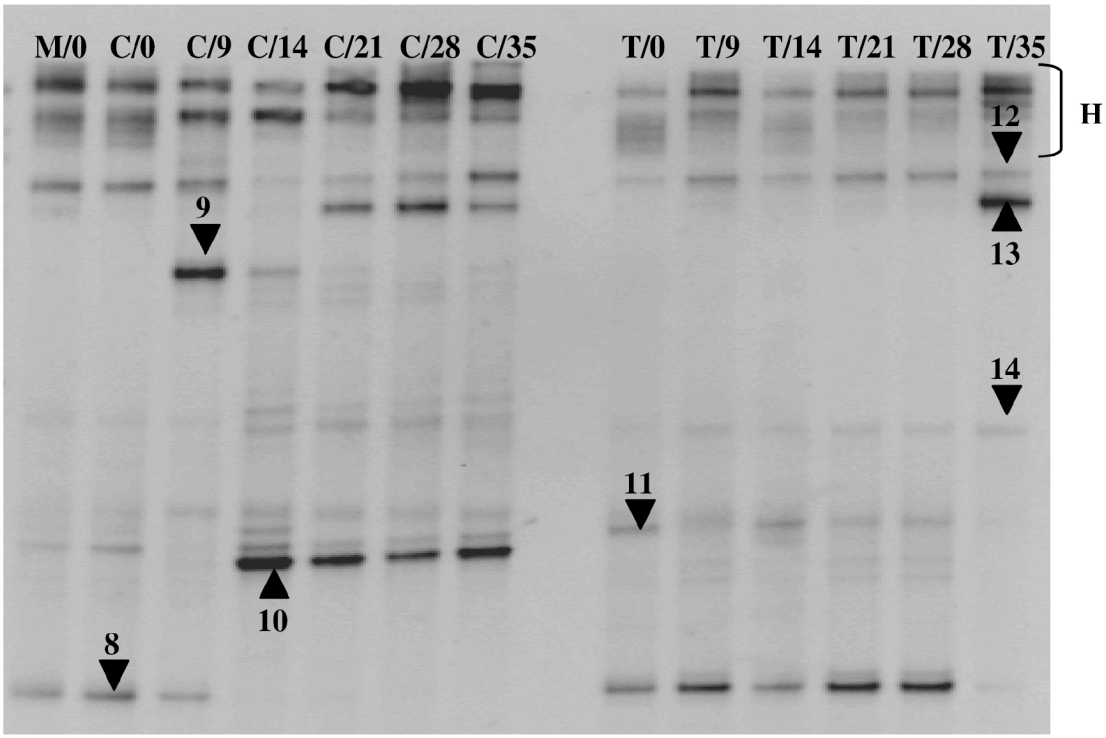

Figure 5.

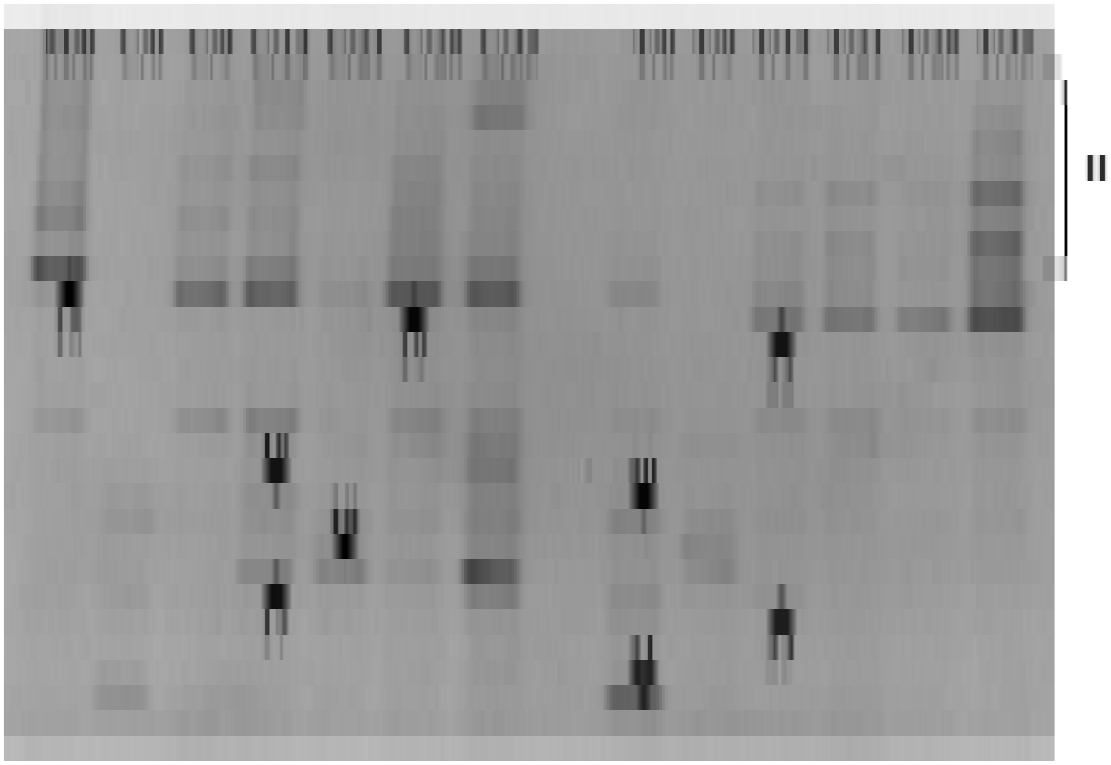
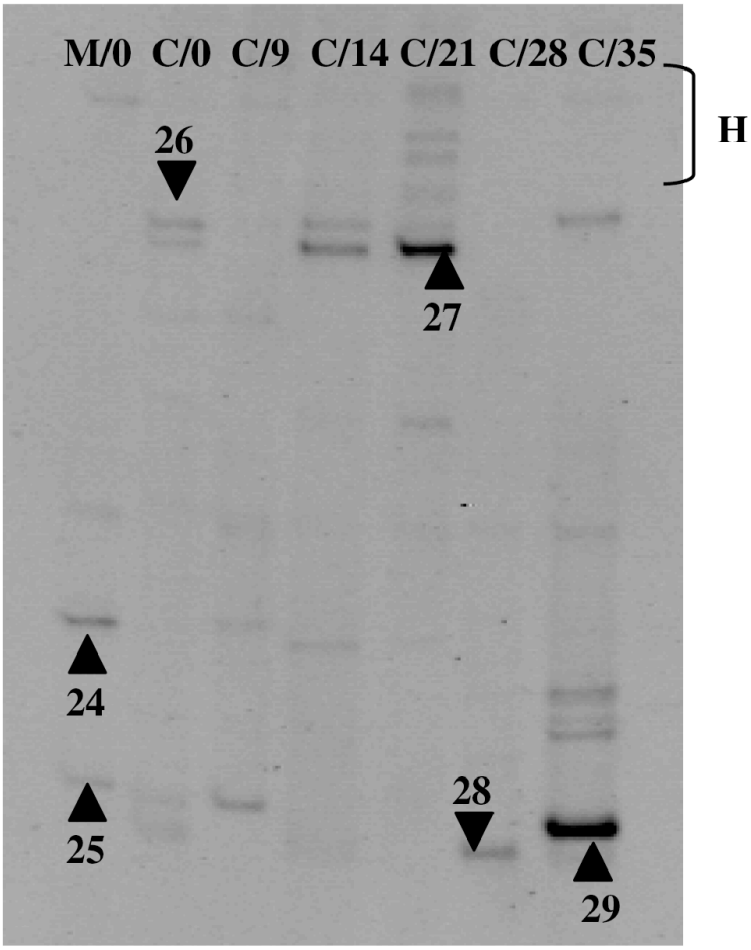
Figure 6.